Winter 2007

# Transesterification of waste olive oil by Candida lipase

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# TRANSESTERIFICATION OF WASTE OLIVE OIL BY CANDIDA LIPASE

BY

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**THESIS**

Submitted to the University of New Hampshire

in Partial Fulfillment of

The Requirements for the Degree of

Master of Science

in

Chemical Engineering

December, 2007

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

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## DEDICATION

I would like to dedicate this work to my son Chen Shen.

## ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Vasudevan for his guidance and support. He has been my source of knowledge for the entire years of my stay in UNH. He is always ready and willing to help me. Thank you, Dr. Vasudevan.

I also would like to thank Dr. Fan and Dr. Barkey for serving as my thesis committee members. I really appreciate their willingness.

Also, I would like to thank my wife and my son for being great support throughout my studies and research. To my friends Maninder, Zhengyu Zhang's family and Yuan Ma's family, I want to express my sincere thanks, thank you for your support either to me or to my family.

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## ABSTRACT

### TRANSESTERIFICATION OF WASTE OLIVE OIL BY CANDIDA LIPASE

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Biodiesel was produced by transesterification of waste olive oil with methanol and Novozym®435.

Experiments were conducted to determine the effect of molar ratio of methanol to triolein, mode of methanol addition (single addition versus stepwise addition), reaction temperature and mixing speed on biodiesel yield. It was found that a molar ratio 9:1 of methanol:triolein provided better result, and stepwise addition of 3:1 * 3 had higher yield than a single addition of methanol at 9:1 molar ratio. The reaction was faster and the yield was much higher at 60°C than at 40°C. However, the mixing speed did not play a significant role in the range selected, 100rpm - 400rpm.

Studies were also performed to evaluate the effect at different acyl acceptors and / or solvents on biodiesel yield. Among the selections, tert-butanol with methanol as the solvent and acyl acceptor gave relatively high yield, while tert-butanol without methanol gave a very low yield. Hexane as the solvent and methanol as the acyl acceptor also gave good yield. While using methyl acetate as both the solvent and the acyl acceptor, the yield was a little low, but still much higher than in tert-butanol without methanol.

However, compared to the above selection, the yield of biodiesel with hexane as the solvent and ethanol as the acyl acceptor was as high as 0.63 g biodiesel / g used oil, while the highest yield in the other cases was about 0.43 g biodiesel / g used oil.

Finally, studies on enzyme efficacy were carried out. The efficacy of Novozym®435 was determined by reusing the enzyme after washing it with solvent hexane. When methanol was added in one shot (at a ratio of 9:1 with respect to triolein), a slight increase in yield was observed in the second run after the initial first run. The yield then started to decrease progressively for each run beyond the second run, but the activity was still high for the first 6 runs. When methanol was added stepwise at 3:1 *3, the enzyme underwent slow irreversible inactivation.

For sugar catalyst, feasibility studies were done on its preparation and the activity testing. The conversion of triolein by sugar catalyst was lower than 1%, which indicated that the sugar catalyst activity was very low.

# CHAPTER 1

## INTRODUCTION

Biodiesel is a clean-burning fuel produced from grease, vegetable oils, or animal fats. Its chemical structure is that of fatty acid alkyl esters. Biodiesel is produced by transesterification of oils with short-chain alcohols or by the esterification of fatty acids. The transesterification reaction consists of transforming triglycerides into fatty acid alkyl ester, in the presence of an alcohol, such as methanol or ethanol, and a catalyst, such as an alkali or acid, with glycerol as a by-product [1]. Chemical reaction at supercritical conditions without the use of a catalyst has also been proposed [2].

In the U.S., oil is the fuel of transportation. Coal, nuclear, hydropower and natural gas are primarily used for electric power generation. The US with 5% of the world's population, consumes 25% of the world's petroleum, 43% of the gasoline and 25% of the natural gas. According to Oil and Gas Journal (O&GJ) estimates, worldwide reserves at the beginning of 2004 were 1.27 trillion barrels of oil and 6100 trillion cubic feet of natural gas. These are proven recoverable reserves. At today's consumption level of about 85 million barrels per day of oil and 260 billion cubic feet per day of natural gas, the reserves represent 40 years of oil and 64 years of natural gas [20].

Thus due to diminishing petroleum reserves and the deleterious environmental consequences of exhaust gases from petroleum diesel, biodiesel has attracted attention during the past few years as a renewable and environmentally friendly fuel. Since

biodiesel is made entirely from vegetable oil or animal fats, it is renewable and biodegradable. Biodiesel also contains very little sulfur, polycyclic aromatic hydrocarbons, and metals. Diesel fuels can contain up to 20% polycyclic aromatic hydrocarbons. For an equivalent number of carbon atoms, polycyclic aromatic hydrocarbons are up to three orders of magnitude more soluble in water than straight chain aliphatics. These compounds are not readily biodegraded, tend to bioaccumulate and are reported to be mutagenic. The fact that biodiesel does not contain polycyclic aromatic hydrocarbons makes it a safe alternative for storage and transportation.

Like petroleum diesel, biodiesel operates in compression-ignition engines. Biodiesel is most often blended with petroleum diesel in ratios of 2 percent (B2), 5 percent (B5), or 20 percent (B20). It can also be used as pure biodiesel (B100). Biodiesel fuels can be used in regular diesel vehicles without making any changes to the engines. It can also be stored and transported using diesel tanks and equipment. Since biodiesel is oxygenated, it is a better lubricant than diesel fuel, increasing the life of engines, and is combusted more completely. Indeed, many countries are introducing biodiesel blends to enhance the lubricity of low-sulfur diesel fuels [3]. The higher flash point of biodiesel makes it a safer fuel to use, handle and store. With its relatively low emission profile, it is an ideal fuel for use in sensitive environments, such as heavily polluted cities. It has been shown that biodiesel offers lower emissions of greenhouse gases compared to petroleum diesel [4]. The amount of particulate matter produced by biodiesel is also considerably less.

There are several technical challenges that need to be addressed to make biodiesel profitable. First, the high cost of virgin vegetable oil and the source of triglycerides play a large role in process profitability. In order to reduce production costs and make it competitive with petroleum diesel, low cost feedstock such as non-

edible oils, waste frying oils and animal fats, could be used as raw materials. However, the relatively higher amounts of free fatty acids and water in this feedstock results in the production of soap in the presence of alkali catalyst. Thus, additional steps to remove any water and either the free fatty acids or soap from the reaction mixture are required. In fact, commercial base-catalyzed processes often employ an acid-catalyzed pre-esterification reactor to remove excess free fatty acids.

Alkali-catalyzed transesterification proceeds much faster than that catalyzed by an acid and is the one most used commercially [5]. Considerable research has been done on biodiesel made from virgin vegetable oils (e.g. soybean oil, sunflower oil, rapeseed oil) using alkali catalysts. The majority of biodiesel today is produced by alkali-catalyzed (e.g. NaOH, KOH) transesterification with methanol, which results in a relatively short reaction time. However, the vegetable oil and alcohol must be substantially anhydrous and have a low free fatty acid content because the presence of water and/or free fatty acid promotes soap formation. The soap formed lowers the yield of esters and renders the downstream separation of the products difficult [5].

Solid catalyst such as sugar catalyst for biodiesel production has been reported. It is made from common sugars and consists of stable sulphonated amorphous carbon. Sucrose or D-glucose is pyrolysed and then sulphonated [17]. The process with sugar catalyst is friendly and economical since the sugar is inexpensive. If it can be proved that the catalytic activity is effective, the application in industrial biodiesel production can be promising.

New biochemical routes to biodiesel production, based on the use of enzymes, have become very interesting [6-11]. Most of the articles published have used a variety of substrates such as rice bran oil, canola, sunflower oil, soybean oil and

castor oil. The results of biodiesel production by transesterification of olive oil using lipase as a catalyst was recently reported [12].

The transesterification reaction is usually carried out in an organic solvent since alcohol and triglycerides are immiscible. Studies have been conducted using various plant oils and solvent mediums. One alternative to organic solvent is the use of supercritical methanol since it provides a medium for the reaction and also acts as an acyl acceptor. However large scale versions of this process would be uneconomical because it is energy intensive. Lipase has been shown to be effective in the transesterification of sunflower oil in a solvent free medium [13]. One problem that arose was the inhibition of the enzyme due to glycerol formation. The use of ultrasonic agitation for a solvent free reaction using soybean oil and a base catalyst has recently been published [14]. They studied the effect of ultrasonic agitation at 28 and 40 kHz on product yield. It has been shown to increase the yield due to increased transport across the phase interface. A number of different acyl acceptors have shown to be effective with lipase as the catalyst. Methanol and ethanol are the most commonly used alcohols. Longer chain alcohols have also been shown to be effective but they provide lower yields than methanol. Recent studies using methyl acetate as the acyl acceptor and soybean oil show that the use of this acyl acceptor does not lead to inhibition of the enzyme [15].

The optimal conditions for transesterification of virgin olive oil were recently reported [12]. Coggon et al. (2007) [16] reported results of experimental studies in which triolein, a compound present in olive oil, was used. Lipase was used as the catalyst for the reaction. Methanol, ethanol, and methyl acetate were considered as the acyl acceptors. The use of a solvent free medium was also studied.

The focus of biodiesel production should be on used vegetable oils. This thesis reports results of such studies with used olive oil, and it is arranged as follows: Chapter 2 reviews the literature in the area of biodiesel research, Chapter 3 presents the Materials and Methods, Chapter 4 discusses the Results and Discussion, and Chapter 5 gives the Conclusions and Recommendations for future work.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Introduction

A majority of the world's fuel for transportation comes from petroleum, but they are non-renewable and are consumed quickly. With oil at high prices, the usage of environment friendly fuels is being encouraged. One of the viable energy sources is biodiesel, which is produced from edible or non-edible vigin or used vegetable oils and fats. The most common method is transesterification.

Biodiesel is completely natural and renewable. It is 100% vegetable oil based, therefore containing no petroleum or other fossil fuels. Besides huge landfill reductions, this environment-friendly fuel reduces tailpipe emissions, visible smoke and noxious odors. Because biodiesel is non-toxic, biodegradable and non-flammable, handling and storage are safer than conventional petroleum diesel fuel.

Biodiesel is the only renewable alternative diesel fuel that actually reduces major greenhouse gas components in the atmosphere. The use of biodiesel will reduce the following emissions:

a) carbon monoxide

b) ozone-forming hydrocarbons

c) hazardous diesel particulate

d) acid rain-causing sulfur dioxide

e) lifecycle carbon dioxide

The production of biodiesel is growing at an astonishing rate. Some important drivers that may lead to a boom of the biodiesel industry are:

a) relatively low feedstock prices

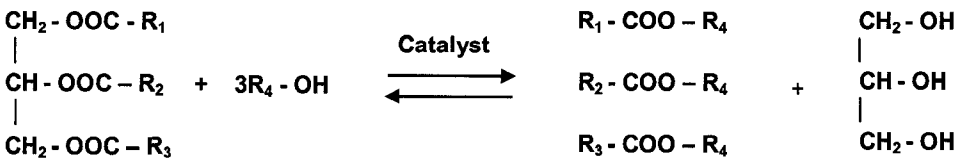
b) environmental concern with diesel fuel

c) reduced dependence on foreign oil.

Currently there are 45 active plants and the National Biodiesel Board expects production to triple this year.

A wide range of sources can be used to produce biodiesel. The raw materials are country specific and depend on the availability of the feed, and most often the plant oils are the sources. The type of fatty acid ester or biodiesel depends on the triglycerides in the sources as the compositions of plant oils vary widely. The commonly plant oils used for biodiesel production are sunflower, safflower, soybean, cottonseed, rapeseed and peanuts. Soy accounts for about 32% of the world's seed production and is the primary plant oil used in the United States. Palm, rapeseed and sunflower seeds are the other major sources for the world's plant oil. Olive oil accounts for about 3% of the world's oil production and is widely grown in the Mediterranean countries, such as Spain.

Biodiesel is a fully renewable liquid fuel source that can be used as an alternative to petroleum diesel fuel. In biodiesel production, a triglyceride (typically a vegetable oil or animal fat) is combined with a simple alcohol (usually methanol) to create biodiesel (methyl esters, made from the combination of fatty acids in the oil and the methanol), with a by-product of glycerin. The generic transesterification reaction is shown as:



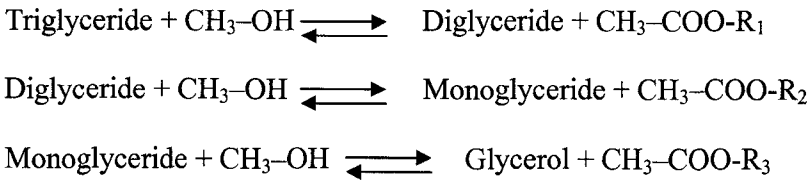

Or, the reaction can be simply summarized by the following transesterification reaction:

1 Triglyceride + 3 Alcohol → 3 Biodiesel + 1 Glycerol

The reaction requires a 3:1 stoichiometric ratio of alcohol to the triglyceride. Excess alcohol is often used.

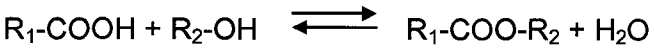
On a weight balance basis for the transesterification reaction depicted above, for every 100 pounds of oil and 10 pounds of methanol added, approximately 100 pounds of the biodiesel and 10 pounds of glycerol are produced.

The overall reaction is reversible and consists of three consecutive reactions and the below is the basic mechanism:




In the reaction, the monoglyceride and diglyceride are the intermediates and the glycerol is the by-product.

There is free fatty acid in the oil sources, especially in the used oil. Instead of tranesterification, the biodiesel is produced through esterification reaction, which is shown as:



In the transesterification reaction, the alcohol acts as acyl acceptor. Very often methanol is used due to its availability and low cost. If methanol is used in the reaction, the fatty acid methyl ester is the primary source of biodiesel. One of the important factors in the reaction is the molar ratio of alcohol to triglyceride. Since the reaction is reversible, the excess amount of alcohol will drive the reaction towards the formation of the biodiesel and glycerol. But too high molar ratio of alcohol will exert inhibition on the enzyme and lower the reaction. Ethanol can also be used as the acyl acceptor. Differ to methanol and ethanol, some chemicals such as methyl acetate can act both as the acyl acceptors and the solvents. Methyl acetate reacts with the triglyceride by a similar mechanism shown before.

Many studies show that the transesterification reaction takes place in the presence of organic solvents. This is a common method adopted as it eliminates the issue of poor miscibility between triglyceride and alcohol. Hexane has been successfully used with a number of difference plant oil sources. Other studies on co-solvent using ultrasonic energy or solvent-free reactions have also been carried out.

## 2.2 Biodiesel production

### 2.2.1 Chemical catalysis

The chemical processes for production of biodiesel are well known, they use base or acid as the catalysts.

Industrially, many biodiesel plants employed the base catalysts. Normally sodium hydroxide and potassium hydroxide are used as the catalysts. The quantity of the catalyst used in the reaction is about 1 weight percent of the plant oil being used.

The base catalyzed reaction works with many plant oils and short chain alcohols. Under atmospheric pressure and 66°C, 98% conversion can be obtained. The reaction takes about 30 minutes, but it highly depends on the mixing and heating of the reactants. Although base catalysis is faster and efficient, the reaction needs to be anhydrous, because the water will cause saponification with the base to form the soap and water, the latter will allow the cycle to continue. This results in not only consuming the base catalyst, but also the difficulties in separating the product biodiesel and the by-product glycerol.

Besides the base catalyst, the acid catalyst is also applied. The transesterification used sulfuric, phosphoric, hydrochloric or organic sulfuric acid as catalyst to produce biodiesel. The acid catalyzed reaction is generally slower than that by base catalyst, depending on the reaction conditions, it takes about 48 to 96 hours to complete the conversion. Although the reaction is slower, it is more suitable to use acid catalyst when the plant oil source has a relatively high free fatty acid and water content because it has the ability to catalyze both the transesterification and esterification reactions. Also, this reaction is performed directly on the oil-bearing material rather than purifying the oil from the source, as the alcohol can act both as the extraction solvent and the esterification agent.

### 2.2.2 Supercritical fluid

Biodiesel can be produced at supercritical fluid without catalyst. This reaction is under condition of high temperature and high pressure, thus it has high requirements on the reaction equipment and consumes energy. But compared to other methods of biodiesel production, supercritical fluid has its advantages and potential

application. Supercritical fluid can increase the reaction surface and solve the problem of product separation. Also, the production is an environmental friendly process, the yield of biodiesel via this method is very high and the solvent can be recycled with simple treatment. So the research on the reaction process and conditions has become hot recently. In the supercritical reaction system, the oil or fat needs no pre-treatment, as the free fat acid and water will not have effect on the reaction [8].

### 2.2.3 Sugar catalysis

To make the process of biodiesel production from vegetable oil more environmental friendly and economical, new efficient solid catalyst is being researched on. Recently, such a catalyst was developed. It is prepared from common sugars and consists of stable sulphonated amorphous carbon. The sugar catalyst is recyclable and the activity is markedly high.

Compared to traditional solid catalysts, such as Nafion, the newly developed sugar catalyst is inexpensive, its activity is stable and will not be lost rapidly. To prepare for this catalyst, the sugar is first heated for 15h at 400 °C under $N_2$ flow to produce a brown-black solid, this pyrolysis process at low temperature leads to incomplete carbonization which forms small polycyclic aromatic carbon rings. Then the solid is heated in fuming sulfuric acid at 150 °C for 15h under $N_2$, this sulphonation process induces the sulphonite group to the rings and generates a stable solid with high density of active sites, enabling high efficient catalytic activity. Structural analysis shows that the sugar catalyst consists sheets of amorphous carbon with hydroxyl group, carboxyl group and high concentration of sulphonite group. After sulphonation and cooling down to room temperature, distilled water is added to

the mixture to form a black precipitate, which is further washed repeatedly in hot distilled water until no impurities are detected [17].

### 2.2.4 Enzyme catalysis

Another promising method for biodiesel production is using enzyme lipase to catalyze the transesterification reaction. The reaction condition is mild, it requires less alcohol, and the products are easy to be collected. Not like chemical catalysis, no pollutant will be drained for this reaction. Also, during the process, other high-value products, such as bio-degradable lubricant and relevant additives, can be further synthesized. However, there are disadvantage by using enzyme. First, the lipase tends to get together and it is not easy to disperse them, which make the catalysis efficiency low, thus there needs some treatments on the lipase. Common treatments include enzyme immobilization, enzyme purification and pre-treatment and enzyme modification, etc. Second, lipase can catalyze the transesterification reaction with long-chain alcohol effectively, but the conversion with short-chain alcohol is low, the short-chain alcohol will impact the enzyme activity and normally require stepwise addition [8].

To get high yield of product, the enzyme must fully contact with the reactants, since oil and alcohol are immiscible to each other, solvent is added to the reaction to solve the problem, but the addition of solvent will impact the separation of biodiesel and the reuse of enzyme.

Lots of studies have been carried out on lipase immobilization. Immobilized lipase is very stable and can be reused for many times, and it is simple to separate the products. But the lipase is expensive, which makes the production cost very high and

restricts its industrial scale application. Recently, whole cell catalyst is studied. The process is just to culture the cells which produce the lipase within the cells, no enzyme purification is required. The whole cell serves as the immobilized enzyme. This can be a potential application in the industry.

### 2.2.5 Novozym®435

Novozym®435 is a commercial lipase and is manufactured by Novozymes, a famous enzyme producer. Novozym®435 comes from Candida Antarctica and is the lipase B. It is obtained from the genetically modified microorganism Aspergillus via submerged fermentation and then is adsorbed onto a macroporous resin for immobilization [18]. Now it is a widely used lipase in bio-catalyzed reactions. It is highly versatile and can catalyze various substrates. Novozym®435 is a thermally stable and robust catalyst, it keeps high activity in many organic solvents. Many researches show that this enzyme is efficient in transesterification catalysis for biodiesel production.

# CHAPTER 3

# EXPERIMENTAL

## 3.1 Materials

Pure, all natural, classic olive oil (Bertoli brand) was the original oil source, it was fried with food and then the oil was collected and filtered as the waste olive oil (prepared by Dr. Vasudevan), which used in the experiments. Lipase B from *Candida antarctica* adsorbed onto a macroporous resin (Novozym®435) was purchased from Sigma-Aldrich (St. Louis, MO). The activity of the enzyme was about 10,000 units/gram. The standards, triolein, oleic acid and oleic acid methyl ester were obtained from Sigma-Aldrich (St. Louis, MO). HPLC grade methanol purchased from Sigma-Aldrich and 200 proof USP grade ethanol were the alcohols used in the reaction. HPLC grade hexane (Sigma-Aldrich) was used as a solvent in the experiments. 99% methyl acetate purchased from Acros Organics was used both as a solvent and as an acyl acceptor. Certified tert-butanol purchased from Fisher Scientific was used both as solvent and as an acyl acceptor.

## 3.2 Methods

### 3.2.1 Analytical method

The transesterification reaction was monitored using a HP 5890 gas chromatograph. A 15 meter RTX-1 column with an inner diameter of 0.32 mm and a film thickness of 3 microns was used in the analysis. A flame ionization detector was used to analyze the samples and its temperature was set at 275°C. The temperature of the injection port was set at 275°C and the oven temperature at 185°C. This method gave the best possible separation. 0.5 μl of the sample was injected into the column. Each run took approximately an hour to complete depending on the reaction of interest. Standards of triolein, methyl oleate and oleic acid were purchased from Sigma-Aldrich. They were used to determine retention times for the compounds of interest and to establish calibration curves.

In all the runs, the yield of methyl oleate (biodiesel) was determined. From the calibration curves, areas were determined with Origin 5.0. For a given sample size β, γ grams of the appropriate solvent were added for dilution. The weight percentage for the ester of interest in the mixed sample, ω, could be determined from the standard curve for that ester and the determined area. The weight percentage of the ester of interest in the bulk solution, κ, is given by

$$\kappa = \omega(\beta + \gamma)/\beta$$

The amount of the ester in solution is equal to the weight percentage in the bulk solution by the mass of the bulk solution. The yield was calculated from the following equation

$$Yield = (M_{ester,t} - M_{ester,t=0})/M_{triolein}$$

where M denotes the mass of the component of interest. Solvent evaporation was taken into account from the mass of the bulk solution.

### 3.2.2 Standards

Standard curves were used to calculate the biodiesel weight percentage and further to calculate the yield in the reaction. From the standard curves, the products or reactants retention time can be determined and a relationship between the area of the peaks and the corresponding weight percentage can be established.

Pure standard methyl oleate was used. Dissolved weighed methyl oleate in selected weighed solvent, hexane, methyl acetate or tert-butanol, to prepare known weight percentage methyl oleate standard solutions in sequence. Injected 0.5 μl standard solutions in GC and calculated peak areas corresponding to each weight percentage. Using the data to get the standard curve thus to establish the relationship between the methyl oleate weight percentage and GC peak area. For ethanol as the acyl acceptor, pure ethyl oleate was used to establish its standard curve.

## 3.3 Experimental setup

All transesterification reaction runs were carried out in a 40 mL glass vial with a screw top cap and septum. The septa consisted of a 3 mm silicone rubber layer with a 0.13 mm PTFE layer on top. The reactor was placed in a shallow water bath in a Pyrex beaker. The agitation speed and water temperature were controlled with a VWR scientific Series 400HPS hot plate and stirrer. Temperature control was

accomplished through a thermocouple that was placed in the water bath and integrated into the hot plate temperature control loop. A 10 mm diameter dual crosshead stir bar was used to stir the solution.

For sugar catalyst preparation, sugar (sucrose and D-glucose) was pyrolysed in the 55312-3 Lindberg tube furnace at various temperatures for different time, such as at 400 °C for 15h. The temperature was controlled by the 58114 Lindberg temperature controller. The pyrolysed product was sulphonated in fuming sulfuric acid in a 500 ml round bottom distillation flask at various temperature for different long time, such as at 150 °C for 15h. It was heated by the TM-812 Glas-Col Apparatus Heater with the 58114 Lindberg temperature controller. The sulphonation took place under total reflux with vapors condensed by circulating water through a condenser. Both pyrolysis and sulphonation were taken place under $N_2$ flow. The brown-black solid after pyrolysis was either grounded to a powder or without grinding. Distilled water was used for washing.

## 3.4 Reaction procedure

### 3.4.1 Transesterification reaction

Experiments were designed and conducted to determine the effects of methanol to triolein molar ratio, addition mode of methanol, temperature and agitation speed on the yield of methyl oleate. In these runs, 1mL of used olive oil was solvated into 4mL of hexane. The experimental conditions were set depending on the runs as following:

a) Effects of methanol to triolein molar ratio: single addition of 6:1, 9:1, 12:1 and 15:1, 60°C, 150rpm, 500 U Novozym®435. The methanol was added at the beginning.

b) Effects of mode of methanol addition: two sets of experiments were carried out. 1) single addition of 9:1, stepwise addition 3:1*3, 4.5:1*2, 40°C, 150 rpm, 500 U Novozym®435. The methanol were added at 0h for single addition 9:1, 0h and 6h for stepwise addition 4.5:1*2, 0h, 6h and 15h for stepwise addition 3:1*3; 2) single addition of 9:1, stepwise addition of 3:1*3, 60°C, 150 rpm, 500 U Novozym®435. The methanol was added at 0h for single addition 9:1, 0h, 14.5h and 23h for stepwise addition.

c) Effects of agitation speed: three sets of experiments were conducted: 1) 150rpm and 400rpm, 40°C, single addition of 9:1 molar ratio with methanol added at the beginning, 500 U Novozym®435. 2) 150rpm and 400rpm, 40°C, stepwise addition of 3:1*3 molar ratio with methanol added at 0h, 6h and 15h, 500 U Novozym®435. 3) 100rpm, 150rpm and 200rpm, 60°C, single addition of 9:1 molar ratio with methanol added at the beginning, 500 U Novozym®435.

d) Effects of temperature: two sets of experiments were carried out: 1) 40°C and 60°C, 150rpm, single addition of 9:1 molar ratio with methanol added at the beginning, 500 U Novozym®435. 2) 40°C and 60°C, 150rpm, stepwise addition of 3:1*3 molar ratio with methanol added at 0h, 6h and 15h, 500 U Novozym®435.

To study the effect of different acyl acceptors and/or solvents on biodiesel yield, hexane with methanol, methyl acetate, tert-butanol with and without methanol,

and hexane with ethanol were used in the reactions. 1ml used olive oil was combined with 4 mL of the solvent or acyl acceptor of interest. For solvent hexane, 3:1*3 stepwise addition of methanol or ethanol were used and added at 0h, 14h and 24h; for acyl acceptor methyl acetate and tert-butanol, no methanol was added; to investigate the effect of methanol on the reaction of acyl acceptor, 3:1*3 stepwise addition of methanol was added to the solvent & acyl acceptor tert-butanol and the methanol were added at 0h, 14.5h and 23h. Other conditions were same as 150rpm, 60°C, 500 U Novozym®435.

The efficacy of Novozym®435 was also determined by reusing the enzyme. 1ml used olive oil was solvated in 4ml hexane, two methods of methanol addition were adopted – single addition of 9:1 with methanol added at the beginning and stepwise addition of 3:1*3 with methanol added at around 0h, 14h and 24h, other conditions were kept constant as 60°C, 150rpm and 500 U Novozym®435. After each run, the liquid in the glass reactor was decanted and the enzyme was washed with hexane for 3 times. The reaction time was 48h.

The temperature was maintained by 40°C or 60°C water bath, the solution was agitated by means of a stir bar. The glass vial reactors were weighed before and after reaction, before and after each sample taking, to detect any possible reactants loss due to evaporation. Also, before taking sample, the glass vial reactors were put in the refrigerator for 5 to 10 minutes to cool the reaction solution, which was also a method to avoid evaporation loss.

### 3.4.2 Sugar catalyst preparation

Experiments were designed to prepare sugar catalyst and then to test its catalysis activity.

a) Sugar catalyst preparation: 20g sucrose was pyrolysed under $N_2$ flow at various conditions, 350°C for 15h, 350°C for 20h, 400°C for 15h and 400°C for 20h; 20g D-glucose was pyrolysed under $N_2$ flow at 400 °C for 15h and 20h. The pyrolysed brown-black solid was ground to powder or without grinding. Then it was sulphonated in 150 $cm^3$ of fuming sulfuric acid. For sucrose product, it was treated for 15h and 20h at 150 °C, for D-glucose, it was sulphonated at 150 °C for 15h. After the sulphonation, the mixture was cooled down to room temperature and 1000 $cm^3$ of distilled water was added to form a black precipitate. The precipitate was separated and washed repeated with 80 °C hot distilled water until all impurities was washed out. Finally, the cleaned precipitate was dried and ready for catalyst use.

b) Sugar catalyst catalysis: the reaction set-up was same as the enzyme catalysis reaction, the sugar catalyst was 0.05g, 0.1g and 0.2g, the reaction temperature was 40 °C, 60 °C and 70 °C, the mixing speed was 200rpm and 400rpm, the methanol was one-step addition 9:1 of methanol to triolein or stepwise addition 3:1*3 of methanol to triolein. Reactant oils involved 65% Glyceryl trioleate, olive oil, used olive oil, methyl acetate and pure oleic acid. Other treatments included with and without sonic treatment, with and without solvent hexane.

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Introduction

In this work, waste olive oil was used for transesterification reactions to produce biodiesel. From the literature review, studies have been conducted with various oils, but not waste olive oil, this was the first time to study waste olive oil as a substrate in a systematic way. Immobilized Novozym®435 was employed in the reaction. The objectives of this research work were:

1. To study the effects of various parameters and thus to determine the optimal conditions for the transesterification of triolein catalyzed by lipase Novozym®435.
2. To identify the effect the different acyl acceptors and / or solvent on biodiesel yield
3. To evaluate the efficacy of the lipase Novozym®435 by reusing the enzyme after washing it with a solvent.

## 4.2 GC analysis

In the reactions, the methyl oleate (biodiesel) was the desired product and the yield of methyl oleate by transesterification reaction from triolein was the parameter

of interest. Instead of using HPLC, gas chromatography was used as the analytical method, because from GC good resolution and separation of products can be obtained, which were the primary objectives when developing the method.

Standards allow to determine the retention time of interested products and to establish the calibration curves. From the chromatogram, the peak area of methyl oleate was determined and the composition of the sample was calculated, thus the yield of biodiesel for a particular reaction can be determined

Figure 4.1 shows that the retention time of methyl oleate is approximately 20 minutes, and it can be stated that the transesterification reaction from triolein and methanol is taking place due to the appearance of a new peak at that time. The ordinate consists of signal from the flame ionization detector in millivolts. The first peak is the solvent and all of it passes through the column within a few minutes. The second peak is methyl oleate.

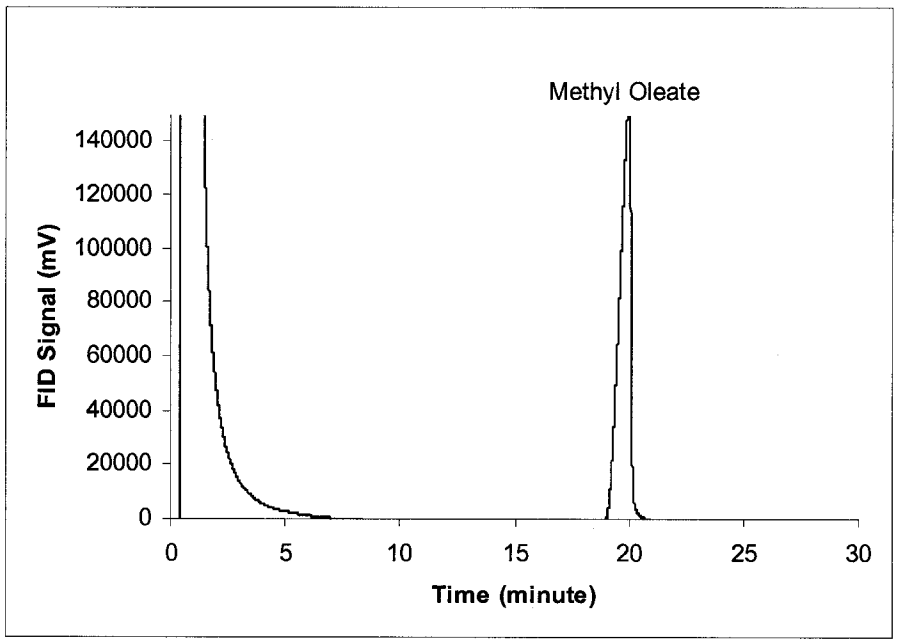



Figure 4.1 Standard chromatogram of methyl oleate.

Figure 4.2 displays typical chromatograms over a 48-hour period. It can be clearly seen that methyl oleate is formed as time progresses. It also shows that several side reactions are occurring because there are other peaks appearing. This indicates that triolein (and oleic acid which comes from triolein hydrolyzation) is not the only compound present in the waste olive oil that reacts with methanol. Other fatty acids such as palmitic and linoleic acids could be present in the waste olive oil as well.

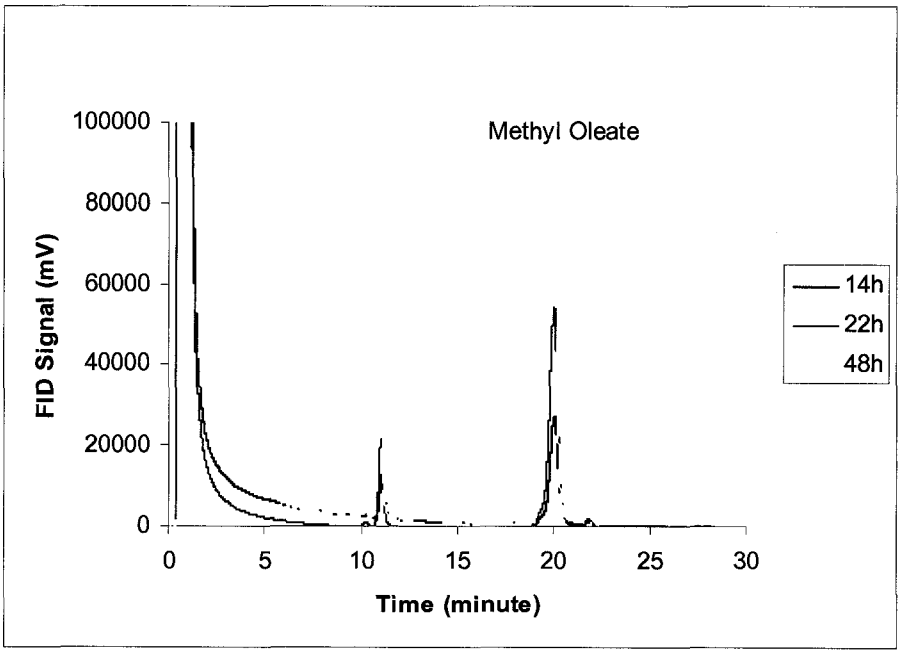



Figure 4.2 Chromatograms demostrating methyl oleate formation.

Appendix A gives the relationship between the weight percentage of methyl oleate in the analyzed sample and its peak area in gas chromatograph. With the equations, one is able to calculate the yield of biodiesel in a particular reaction.

## 4.3 Thermodynamic analysis

For the reaction in the experiments, methanol was used as the alcohol, triolein was the source of triglyceride, and the products were methyl oleate, with glycerol as the by-product. So the reaction was:

$$3\ \text{Methanol} + 1\ \text{Triolein} \underset{\longleftarrow}{\overset{\text{Enzyme}}{\longrightarrow}} 1\ \text{Glycerol} + 3\ \text{Methyl Oleate}$$

From Aspen Property Data, the data for standard Gibbs-energy and standard enthalpy of pure components at 298K are:

$G^{\circ}_{\text{Methanol}} = -162320$ J/mol

$G^{\circ}_{\text{Triolein}} = +1.72\text{E}+7$ J/mol

$G^{\circ}_{\text{Glycerol}} = -447100$ J/mol

$G^{\circ}_{\text{Methyl Oleate}} = -117000$ J/mol

$H^{\circ}_{\text{Methanol}} = -200940$ J/mol

$H^{\circ}_{\text{Triolein}} = -1840000$ J/mol

$H^{\circ}_{\text{Glycerol}} = -577900$ J/mol

$H^{\circ}_{\text{Methyl Oleate}} = -626000$ J/mol

So the standard Gibbs-energy change of the reaction is:

$$\Delta G^{\circ} = G^{\circ}_{\text{Glycerol}} + 3 * G^{\circ}_{\text{Methyl Oleate}} - G^{\circ}_{\text{Triolein}} - 3 * G^{\circ}_{\text{Methanol}}$$

$$= -447100 - 3 * 117000 - 17200000 + 3 * 162320$$

$$= -17511140 \text{ J/mol}$$

The standard enthalpy change of the reaction is:

$$\Delta H^{\circ} = H^{\circ}_{\text{Glycerol}} + 3 * H^{\circ}_{\text{Methyl Oleate}} - H^{\circ}_{\text{Triolein}} - 3 * H^{\circ}_{\text{Methanol}}$$

$$= -577900 - 3 * 626000 + 1840000 + 3 * 200940$$

$$= -13080 \text{ J/mol}$$

The Equilibrium constant K is:

$K = \exp [-\Delta G^0/(R*T)]$

$= \exp [+ 17511140/(8.314*298)]$

$= \exp (7068)$

where R is Universal gas constant and T is 298K

So K is extremely large.

$K = (C_{glycerol} * C^3_{methyl\ oleate}) / (C_{triolein} * C^3_{methanol})$

where C is the concentration of the components in the reaction.

Thus the reaction is almost 100% complete at the equilibrium state.

$\ln(K/K') = (- \Delta H^0/R) * (1/T - 1/T')$

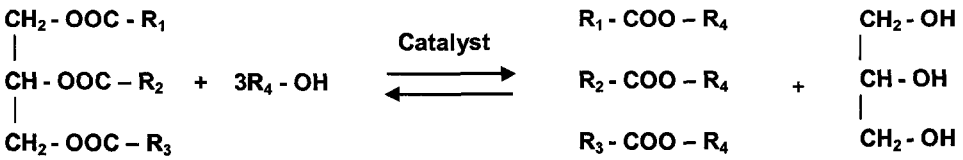
From the K at 298K, K' at 313K and 333K can be calculated, which are also extremely large and the reactions are almost 100% completed at each equilibrium state.

Since the standard enthalpy change of the reaction is negative, it is an exothermic reaction.

## 4.4 Enzyme catalysis

### 4.4.1 Effect of molar ratio of methanol to triolein

Methanol plays a critical role in this reaction and its concentration has a considerable effect on the rate at which transesterification proceeds. In general, the reaction proceeds according to the following stoichiometry:




The stoichiometry of the transesterification reaction requires 3 moles of alcohol to one mole of triglyceride and it yields 3 moles of fatty esters and 1 mole of glycerol. Even though methanol is a reactant, it also inhibits the enzyme [6] [8]. Previous research indicates that in mixtures containing more than 3 molar equivalents of methanol with respect to triolein, Novozym®435 starts to show deactivation [8]. However, too small a ratio of methanol-triolein can result in lower yield, also the presence of other fatty acids in waste olive oil, namely, palmitic and linoleic acids, will consume methanol, Figure 4.2 shows the existence of side reactions. One of the earlier experiments showed that the yield was very low in the reaction with a molar ratio 3:1 of methanol to triolein.

Figure 4.3 shows biodiesel yield for different methanol to triolein molar ratios. The reaction temperature is 60C and the methanol is added in one-step at the beginning of the reaction. The ratio of methanol to triolein was kept higher than the stoichiometric ratio of 3:1.

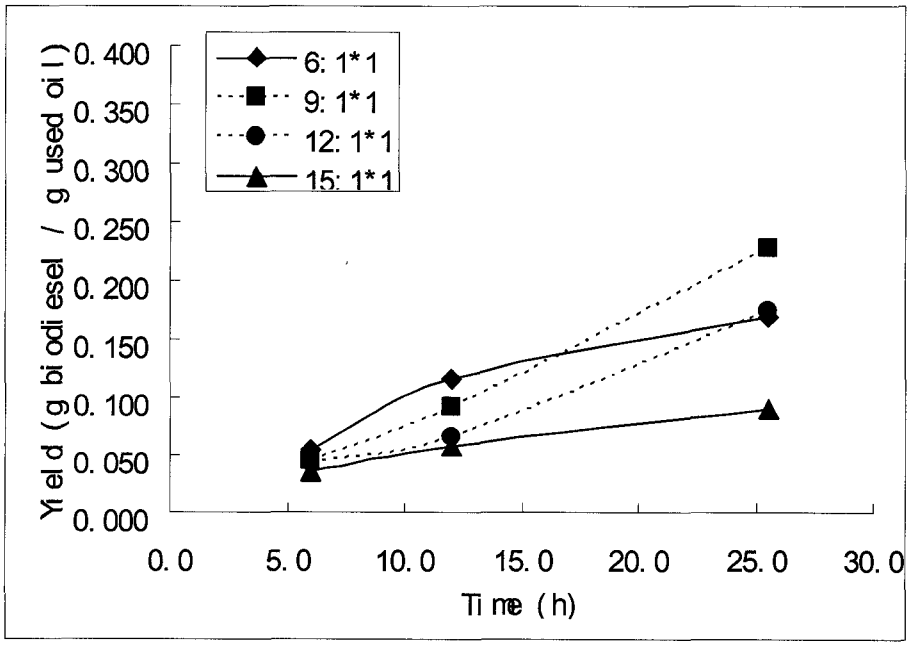



Figure 4.3 Effect of molar ratio of methanol to triolein on yield. Conditions: 60°C, 150 rpm, 500 U enzyme

As the ratio of methanol: triolein is increased, the initial reaction rate decreases which can be seen from the decrease of the slopes suggesting that Novozym®435 is inhibited by methanol. These results are consistent with what was observed for trials with virgin olive oil [12]. However, the yield of methyl oleate increases first to reach a maximum at the ratio of 9:1, then decreases. It is possible that the enzyme deactivation by methanol is reversible, at the beginning, the methanol concentration is higher than the level of enzyme inhibition, as the reaction proceeds, the methanol is consumed and its concentration decrease and the enzyme undergoes its activity recovery. Since there are other compounds in the waste olive oil that also react with methanol, for the 6:1 molar ratio, although already higher than the stoichiometric ratio, the methanol is still not enough for the reaction to convert all the triolein, so its yield of methyl oleate is lower than the yield in the reaction with a

molar ratio of 9:1. However, when the molar ratio is higher and the methanol concentration is beyond a limit, the enzyme starts to deactivate irreversibly. When the molar ratio reaches 12:1, the enzyme has partial irreversible deactivation, so the yield of methyl oleate is lower than the ratio of 9:1. When the molar ratio of methanol to triglyceride is 15:1, the enzyme is almost irreversibly deactivated.

From Figure 4.4, one can see that when the molar ratio of methanol to triolein is over 9:1, the yield drops, it indicates that irreversible enzyme deactivation takes place rapidly.

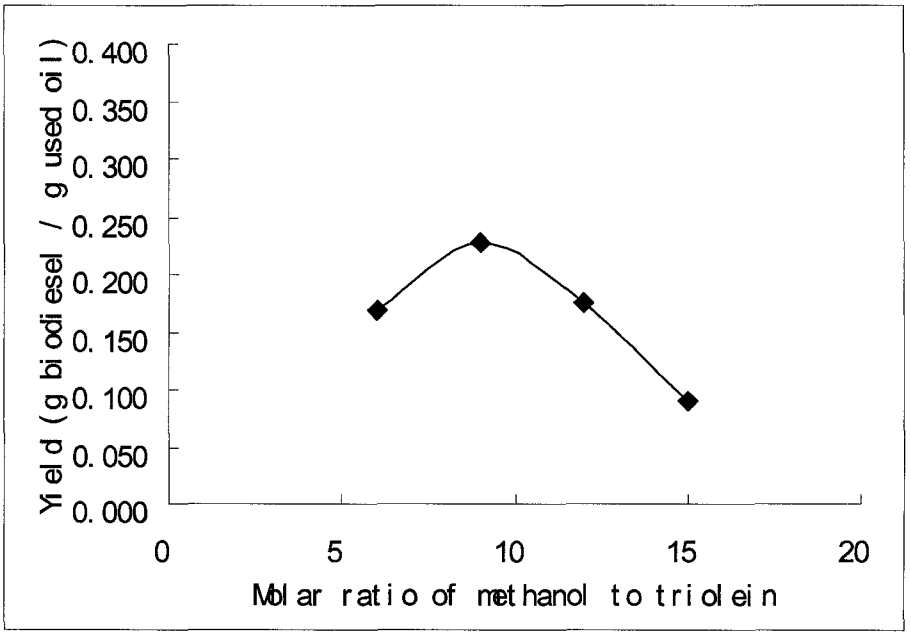



Figure 4.4 Effect of molar ratio of methanol to triolein on yield. Conditions: 60°C, 150 rpm, 500 U enzyme

With used olive oil, the feed also contains free fatty acids and in the presence of methanol, methyl oleate is produced according to the following esterification reaction

$$R1\text{-}COOH + R2\text{-}OH \leftrightarrow R1\text{-}COO\text{-}R2 + H2O$$

The free fatty acid content was about 5%. The formation of water in the esterification step leads to further hydrolysis of the olive oil followed by esterification. Ultimately, this results in phase separation, a phenomenon observed in all the product samples. This was not observed in the runs with virgin olive oil. However, the advantage of the enzymatic approach over a base catalyst is the absence of soap formation. The highest yield obtained with the one-step addition at the end of the reaction period of 25 h was 0.23 with a methanol: triolein ratio of 9:1.

### 4.4.2 Effect of step-wise addition of methanol

For one-step addition, the high methanol concentration will inhibit the enzyme, but lack of methanol can be just as undesirable as enzymatic deactivation. Thus, stepwise addition of methanol should be a promising alternative, because such arrangement can maintain a low enough methanol concentration to avoid enzymatic inhibition or denaturation while providing an overall sufficiently large amount of alcohol to obtain high methyl oleate yield.

Figure 4.5 shows the results of step-wise addition for 3 different ratios of methanol: triolein (3:1, 4.5:1 and 9:1). These runs were carried out at a reaction temperature of 40C and methanol was added in the beginning (in all three cases), and after 6h (for the 4.5:1 and 3:1 ratios) and 15 h (for the 3:1 ratio), respectively. It is evident from the figure that the highest yield is obtained at a methanol: triolein ratio of 3:1. At ratios higher than this, the yields are lower probably due to inhibition of the enzyme by methanol. When the molar ratio is higher, the reaction rate and yield tends to be lower.

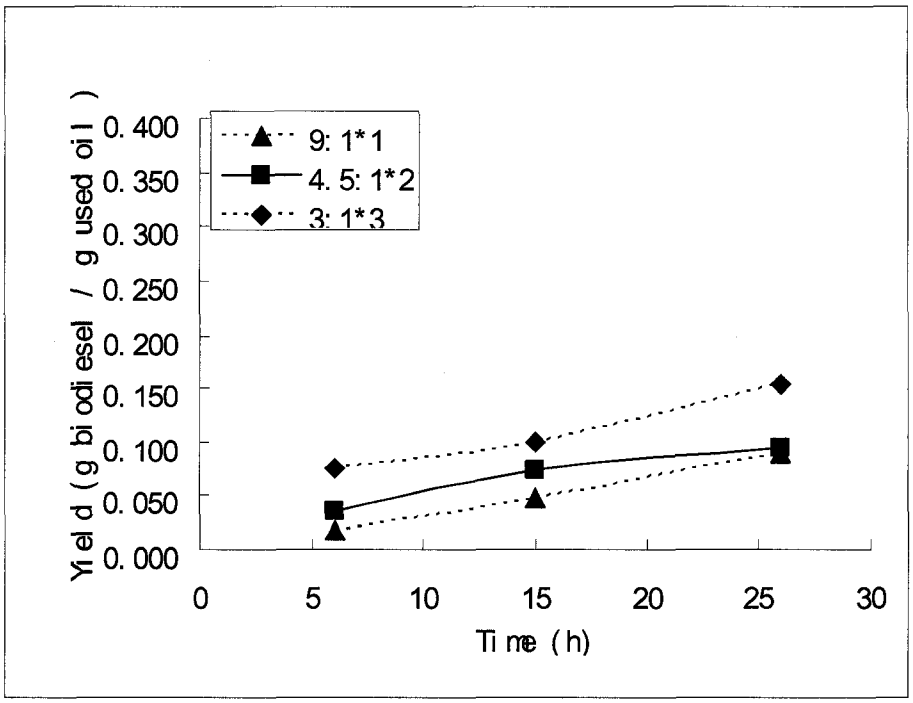



Figure 4.5 Effect of step-wise addition of methanol on yield. Conditions: 40°C, 150 rpm, 500U enzyme, 9:1 ratio

Figure 4.6 compares results of step wise addition (3:1 ratio of methanol: triolein with methanol being added in the beginning, and after 14.5 and 23 h, respectively) with single addition of methanol (methanol: triolein ratio of 9:1) at a reaction temperature of 60C. It is clear from the figure that step-wise addition of methanol results in higher yields throughout; the final yield of methyl oleate after 48 h is 0.4 for step-wise addition compared to 0.25 for single addition. Thus step-wise is a promising method for used oil as it minimizes enzymatic denaturation or inhibition thereby resulting in a higher yield of biodiesel at the end of the reaction period of 48h. It is also noteworthy that the conversion of triglycerides and fatty acids is almost complete at the end of the reaction period.

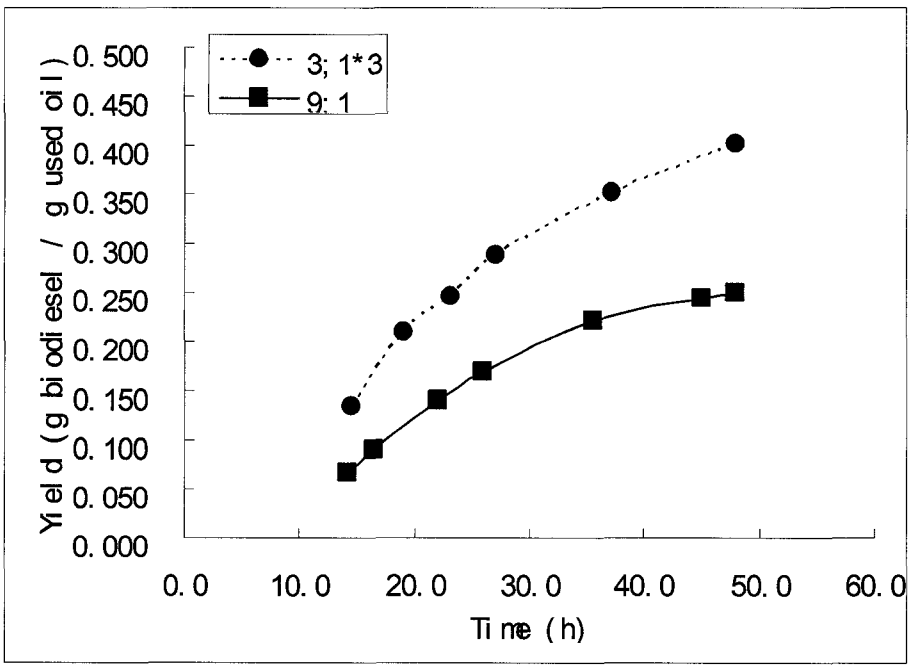



Figure 4.6 Effect of step-wise addition of methanol on yield. Conditions: 60°C, 150 rpm, 500U enzyme, 9:1 ratio

### 4.4.3 Effect of mixing speed

Diffusional resistances may be observed at different levels while working with immobilized enzymes, depending on the nature of the support material that whether it is porous or nonporous and depending on the hydrodynamical conditions surrounding the support material. In other words, mass transfer limitations can be internal and / or external. Due to the nature of Novozym®435, only external mass transfer needs to be considered.

To evaluate the external mass transfer limitation in the transesterification reaction, experiments were designed at various mixing speeds. If diffusional limitation exists, reaction rate and yield of methyl oleate would increase as the mixing speed increases, because when the agitation becomes more intense, the concentration

of reactants at the surface of the immobilized enzyme would increase and approach the bulk concentration and thus the external mass transfer limitation could be lowered or even eliminated.

Figure 4.7 shows the effect of mixing speed (over the range of 150-400 rpm) on biodiesel yield, the reaction is under temperature of 40°C with one-step addition of methanol at the beginning for the ratio 9:1, it is evident that the biodiesel yield is independent of mixing speed.

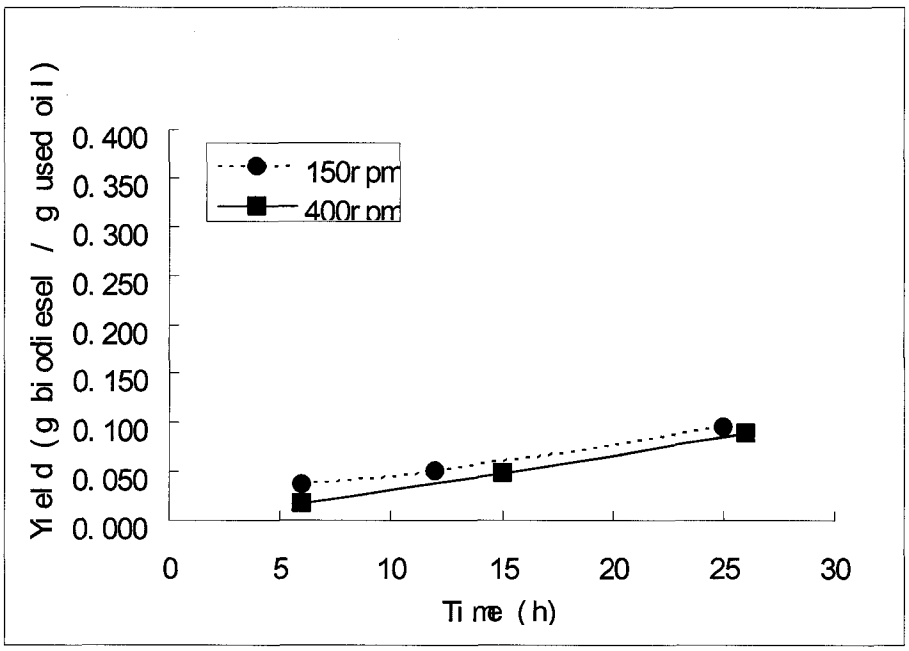



Figure 4.7 Effect of mixing speed on yield. Conditions: 40°C, 500U enzyme, 9:1 ratio

At a higher temperature of 60°C, with the same one-step addition at the beginning, the conclusions are identical, namely, the mixing speed over the range 100-200 rpm does not appear to have a significant influence on the yield. Thus

external mass transfer resistances are not significant enough to adversely affect the rate of reaction. Figure 4.8 displays the effect of mixing speed at a temperature of 60°C with one-step addition of methanol.

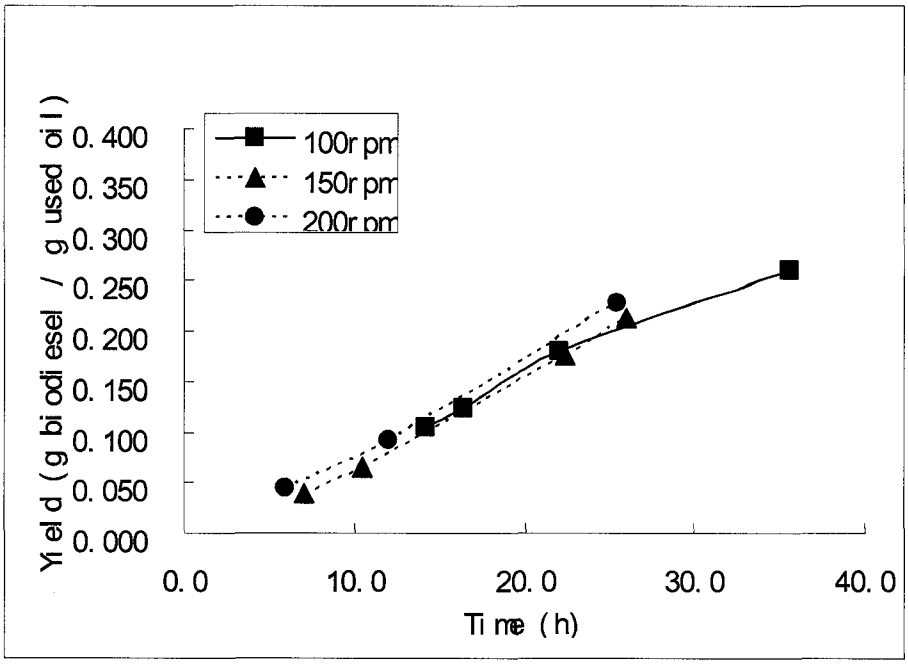



Figure 4.8 Effect of mixing speed on yield. Conditions: 60°C, 500U enzyme, 9:1 ratio

Figure 4.9 gives the results for stepwise addition of methanol. When methanol is added stepwise (to reduce enzyme inhibition by methanol), it is evident that the yield of methyl oleate is unaffected by mixing speed over the range 150-400 rpm.

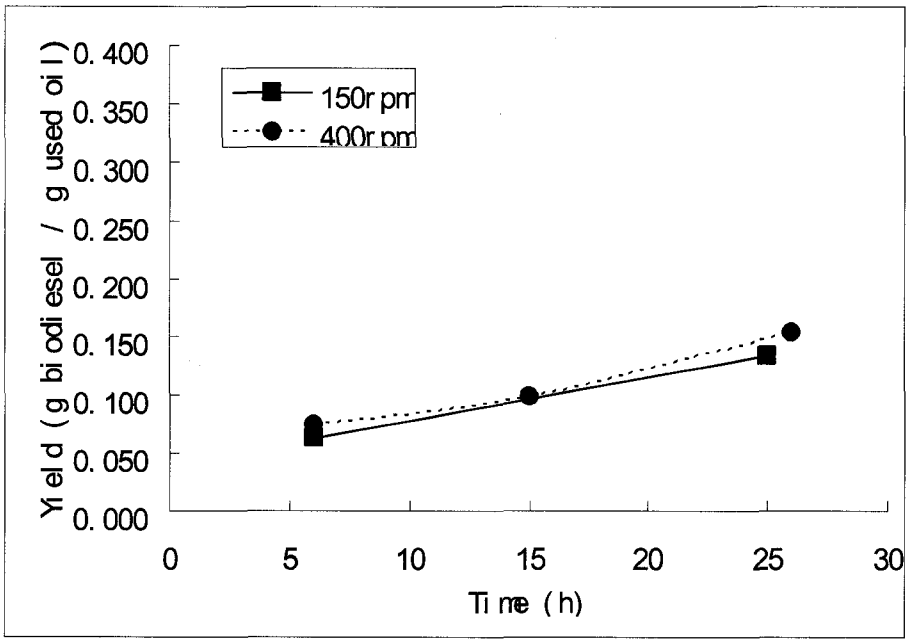



Figure 4.9 Effect of mixing speed on yield. Conditions: 40°C, 500U enzyme, stepwise 3:1*3

From above analysis and results, it can be concluded that external mass transfer resistance is insignificant and that all runs can be conducted at a mixing speed of 100 or 150 rpm to reduce possible enzyme deactivation due to shear stress.

### 4.4.4 Effect of temperature

Both reaction rates and deactivation rates are enhanced by temperature and both these rates have an Arrhenius dependence. During the ascending reaction rate phase (temperature activation), the applicable Arrhenius equation is as following [19]:

$v = k_2 * [E]$

$k_2 = A * e^{-Ea/RT}$, where

v - the enzyme catalyzed reaction rate

[ E ] - the active enzyme concentration

A - the pre-exponential factor

$E_a$ - the activation energy

R - the activation energy

T - the absolute temperature

The descending region is known as temperature inactivation (thermal denaturation). Its kinetics equation can be expressed as [19]:

$$v = - d[ E ] / dt = k_d * [ E ]$$

$$[ E ] = [ E_0 ] * e^{-kd * t}$$

$$k_d = A_d * e^{-Ea/RT}$$

$v = A_d * e^{-Ea/RT} * [ E_0 ] * e^{-kd * t}$, where

$[ E_0 ]$ – the initial enzyme concentration

$k_d$ – the denaturation constant

The optimal temperature is the comprehensive result of the activation reaction and inactivation reaction. It is specific to each reaction catalyzed by a certain enzyme. The effect of temperature on biodiesel yield was investigated and the results are shown in Figure 4.10 for single addition and Figure 4.11 for stepwise addition of methanol.

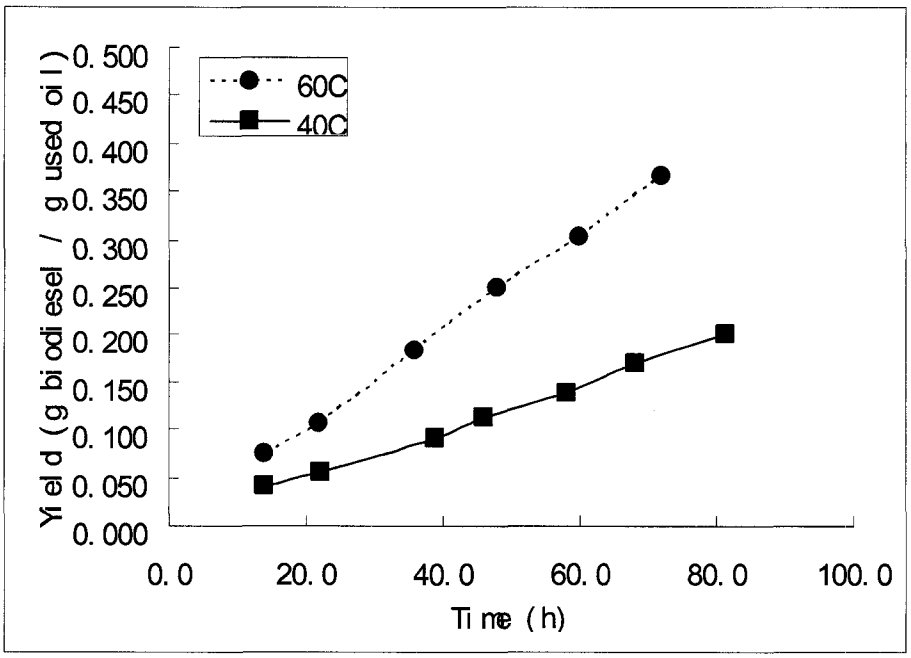



Figure 4.10 Effect of temperature on yield. Conditions: 150rpm, 500U enzyme, 9:1 ratio

The stepwise addition runs were conducted for only 24 hours whereas the trials with a single addition of the acyl acceptor were carried out over nearly 3 days. The transesterification reaction rate and hence the yield of biodiesel is significantly higher at the higher temperature. Novozym®435 is an immobilized enzyme and is known to be thermally stable and robust. In their trials with virgin olive oil, Sanchez and Vasudevan (2006) [12] reported that the enzyme gets deactivated at 70°C. Furthermore, it is not economical to carry out the reaction at 70°C due to the higher energy requirement and loss of solvent by evaporation.

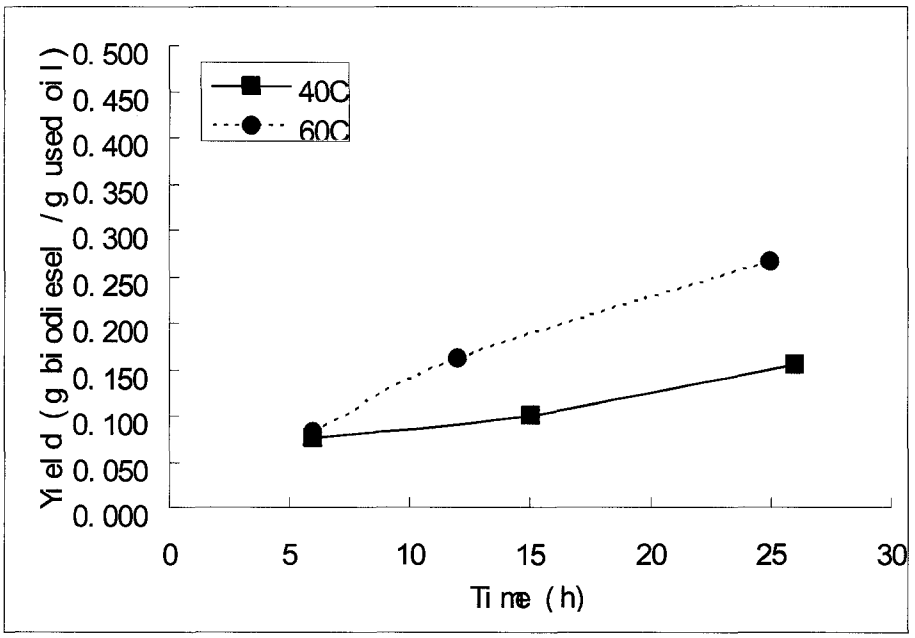



Figure 4.11 Effect of temperature on yield. Conditions: 150rpm, 500U enzyme, stepwise 3:1*3

Another interesting observation in both figures is the relatively small difference in yields at the beginning of the reaction followed by a much larger difference in yields at the two temperatures as the reaction proceeds. This may be attributed to the higher reaction rate at 60°C leading to a faster consumption of methanol, which in turn results in lower enzyme inhibition. For step-wise addition, there is practically no enzyme inhibition until 6 hours of reaction as the molar of methanol to triolein is 3:1.

### 4.4.5 Effect of solvent and acyl acceptor

In the transesterification reaction, solvent is used. Waste olive oil and methanol are immiscible to each other and methanol forms drops that will exert

significant inhibition effect to enzyme. When solvent that is soluble with waste olive oil is added, methanol will diffuse to the solvent and this will alleviate the inhibition to the enzyme by methanol. While some solvents such as hexane can only serve as solvents, others can serve both as solvents and acyl acceptors. To determine the effect on the yield of methyl oleate by different solvents and / or acyl acceptors, hexane, methanol, ethanol, methyl acetate and tert-butanol were selected and five sets of experiments were designed.

The effects of the acyl acceptor and solvent on yield are examined in Figure 4.12. In some cases, the acyl acceptor also acted as the solvent and hence there was no need to add a different solvent. Tert-butanol and methanol obtained a relative high yield, with tert-butanol acting both as the acyl acceptor and solvent. In contrast, the yield at the end of the run was lower when methanol was used as the acyl acceptor in conjunction with hexane. The yield with tert-butanol alone was significantly lower. When methanol is added, there appears to be some synergy. It was observed that oil was better dispersed in a mixture of butanol and methanol. It is quite likely that methanol is a good acyl acceptor and that the presence of tert-butanol limits the inhibition of the enzyme by methanol. It is also interesting to note that methyl acetate acts both as the acyl acceptor and solvent and that the yields are lower, but not significantly lower than with methanol/hexane. However, compared to above selections, the combination of ethanol as the acyl acceptor and hexane as the solvent gave much higher yield. It is possible that ethanol is a better acyl acceptor and also ethanol exerts less inhibition on the enzyme.

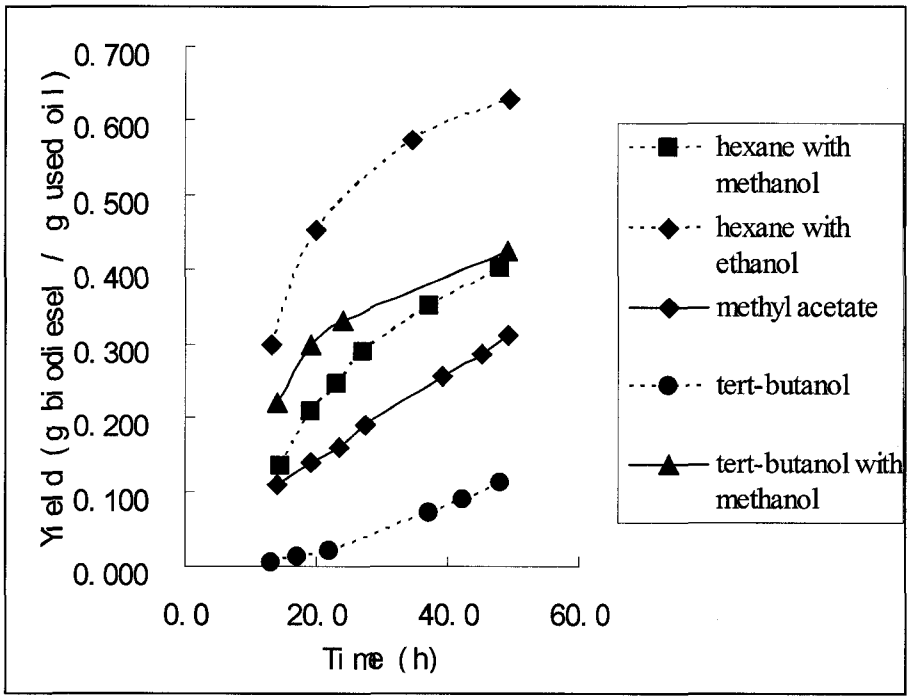



Figure 4.12 Effect of solvent and acyl acceptor on yield. Conditions: 60°C, 150rpm, 500 U enzyme, stepwise addition 3:1*3 for methanol

It was also observed that excessive solvent does not result in improved yields. In our case, identical yields were obtained when the amount of hexane was 3, 4 or 5 mL in the trials with methanol as acyl acceptor.

### 4.4.6 Enzyme reutilization

Enzymatic transesterification is a promising biochemical method for producing biodiesel. But compared to chemical catalysis, one drawback of enzyme catalysis is the expensive cost of the catalyst, which leads to the idea to test the efficacy of the enzyme. If the enzyme can be re-used for many times without significant loss of activity, the cost of biodiesel production will decrease greatly.

When methanol is used as the acyl acceptor, glycerol is one of the major by-products. As it forms, it will stick to the enzyme and has serious negative effects on the enzyme activity. Thus after each run, the glycerol needs to be removed. In the following experiments, hexane was used to wash the enzyme for three times to remove the glycerol after each batch.

Novozym®435 is expected to be resistant to denaturation because its immobilization makes it a particularly robust and stable enzyme. In this research, successive runs were performed to study the endurance of Novozym®435 with respect to the number of batches used. A brand new reactor load, consisting of 1 mL of oil and 144 μL of methanol solvated in 4 mL of hexane, was introduced every 48 hours. The same enzymatic load, 500 U, was maintained throughout these experiments. After each run, the solvent and acyl acceptor were decanted, and the enzyme was washed three times with hexane.

The results of enzyme reutilization for both single and stepwise addition of methanol are shown in Figures 4.13 and 4.14, respectively. When methanol is added in one shot (at a ratio of 9:1 with respect to triolein), a slight increase in yield is observed in the second run after the initial first run. The yield then starts to decrease progressively for each run beyond the second run. This is shown in Figure 4.13. The enzyme particles appeared to be slightly swollen after the first run, but the increase in yield cannot be attributed to this (or to reduction in mass transfer resistance.) Furthermore, such an increase in yield beyond the first run (or in the second run specifically) is not observed when methanol is added step wise (see Figure 4.14).

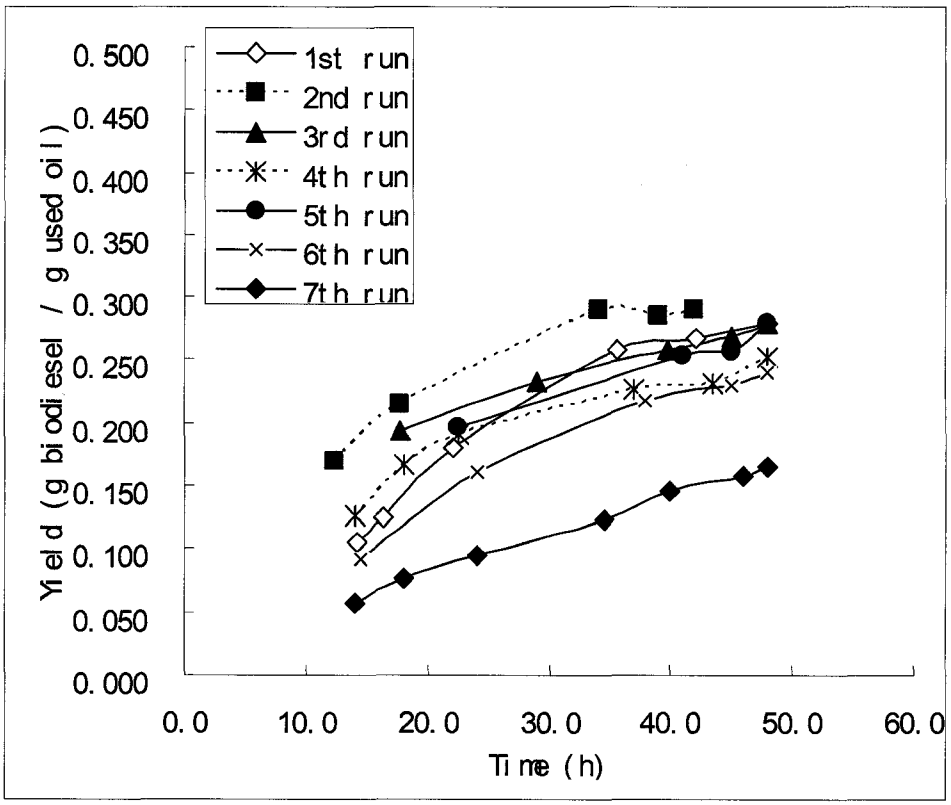



Figure 4.13 Effect of enzyme reutilization on yield. Conditions: 60°C, 100 rpm, 500 U enzyme, 9:1 ratio

The slight increase in yield in the second run in one shot addition of methanol is also not an experimental artifact as we were able to replicate the results repeatedly. It is possible that the enzyme undergoes reversible inhibition by methanol and that all the activity is recovered when the enzyme is washed with hexane at the end of the first run. The increase in activity may perhaps be due to conformational changes that the enzyme undergoes, which makes the active sites more accessible to the reactant. At the same time, the enzyme also undergoes slow irreversible inactivation, which may be compounded by the periodic addition of methanol in stepwise addition.

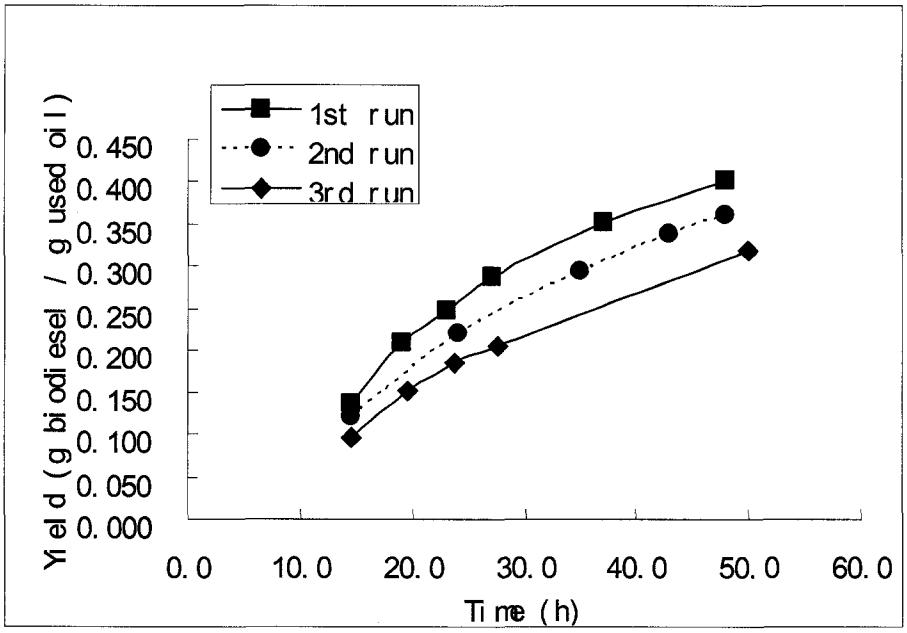



Figure 4.14 Effect of enzyme reutilization Conditions: 60°C, 100 rpm, 500 U enzyme, stepwise addition 3:1*3

Although the enzyme activity is reduced slightly at the end of every run due to enzyme deactivation, the activity is still high for the first 6 runs. The productivity of the enzyme at the end of each run for the two modes of addition was calculated. Table 4.1 shows the results for the single-addition mode. The productivity remained more or less constant during the first 6 runs when methanol is added in one shot at the beginning of the reaction. The average productivity was $9.7 \times 10^{-6}$ g biodiesel/U-h during the first 6 runs. A noticeable drop in productivity was seen at the end of the $7^{th}$ run, with the value dropping to $5.7 \times 10^{-6}$ g biodiesel/U-h. In the case of step-wise addition of methanol, the productivity was substantially higher compared to the 'single-addition' runs, with an average value of $1.3 \times 10^{-5}$ g biodiesel/U-h.

| Batch | Yield (g biodiesel / g used oil) | Productivity (g biodiesel / U enzyme-h) |
|---|---|---|
| 1 | 0.280 | $9.80 * 10^{-5}$ |
| 2 | 0.290 | $11.57 * 10^{-5}$ |
| 3 | 0.249 | $9.76 * 10^{-5}$ |
| 4 | 0.253 | $8.92 * 10^{-5}$ |
| 5 | 0.280 | $9.83 * 10^{-5}$ |
| 6 | 0.240 | $8.32 * 10^{-5}$ |
| 7 | 0.165 | $5.73 * 10^{-5}$ |

Table 4.1 Effect of enzyme reutilization on yield and productivity. Conditions: 60°C, 100 rpm, 500 U enzyme, 9:1 ratio

## 4.5 Sugar catalysis

Self-prepared sugar catalyst activity was tested by using it to catalyze the transesterification reactions to produce biodiesel. Other research showed that the sugar catalyst had high catalysis activity. But the research indicated that the sugar catalyst had very low activity. Compared to enzyme catalysis of over 90% biodiesel yield, the yield of biodiesel with sugar catalyst was less 1%, except for the reactant of pure oleic acid where the biodiesel yield was just above 1%.

By varying the conditions of sugar catalyst amount, mixing speed, reaction temperature, with and without solvent, different oils, single-addition and stepwise addition of methanol, with and without sonic treatment, the yields of biodiesel

changed somewhat, but the results were not consistent. It is possible that the conversions are so low that the analysis errors will affect the results.

During the experiments, it was found that the sugar catalyst began to aggregate and attach to the glass vial reactor when the methanol concentration reached to a certain level, which would significantly affect the reaction, so stepwise addition of methanol might be better. However, this approach was not seen to be feasible.

# CHAPTER 5

# CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Conclusions

### 5.1.1 Biodiesel production with Novozym®435

Biodiesel was produced by transesterification of waste olive oil with methanol and Novozym®435. There are many factors that affect the manner in which the transesterification reaction proceeds. Experiments were carried out to investigate the influence of the molar ratio of methanol to triolein, mode of methanol addition, reaction temperature and mixing speed on biodiesel yield.

For waste olive oil, the experiments results indicated that a molar ratio of 9:1 for methanol to triolein resulted in the highest biodiesel yield. This ratio is higher than the stoichiometric ratio of 3:1 probably due to the presence of other fatty acids in the feed and due to the fact that waste oil was used. At ratios higher than 9:1, the yield became lower due to enzyme deactivation by methanol.

Stepwise addition of methanol resulted in higher yields of biodiesel probably due to less inhibition of the enzyme by methanol. Higher yields of biodiesel were also obtained at a reaction temperature of 60°C, which resulted in higher reaction rates and lower inhibition of the enzyme active sites by methanol. Mixing speed in the range 100-400 rpm had relatively little effect on the yield. The effect of different acyl acceptors and/or solvents on biodiesel yield was also evaluated. The highest yields

were obtained with ethanol acting as the acyl acceptor and hexane acting as the solvent perhaps due to less inhibition exerted by ethanol on the enzyme and a better acyl acceptor of ethanol.

The efficacy of Novozym®435 was also determined by reusing the enzyme after washing it with a solvent. The results showed that enzyme was very stable and still retained a high activity after several runs. Stepwise addition of methanol resulted in higher overall productivity of biodiesel.

### 5.1.2 Biodiesel production with sugar catalyst

Sugar catalyst was prepared from D-glucose and sucrose by first pyrolysis and then sulphonation.

Unlike the runs with enzyme, the sugar catalyst had very low catalyst activity. The yields of biodiesel were less than 1% except for the pure oleic acid whose yield of biodiesel was just above 1% (can be over 90% for enzyme catalysis). The results were inconsistent with respect to variation in reaction conditions. Since the reaction conversions were very low, errors in analysis affected the results.

## 5.2 Recommendations

Based on the research and the results, the following recommendations are made for future work:

a) Results showed that stepwise addition of methanol is superior than one-addition, it will be worthy to investigate the results by continuous feeding of methanol.

b) The immobilized enzyme was at the bottom of the glass vial reactor, the mixing stirrer always impinged on the enzyme and the enzyme particles were crushed to powder, which could lower the enzyme activity. So solution for avoiding this will enhance the enzyme activity, it will be especially useful in the case of enzyme reuse.

c) The study of synergy of solvent and / or acyl acceptor will be of interest.

d) For reutilization of enzyme with single addition of methanol, it is interesting to explore the phenomenon of yield increase after the first batch.

e) Suitable internal standard can be added to further improve the accuracy of the analytical technique.

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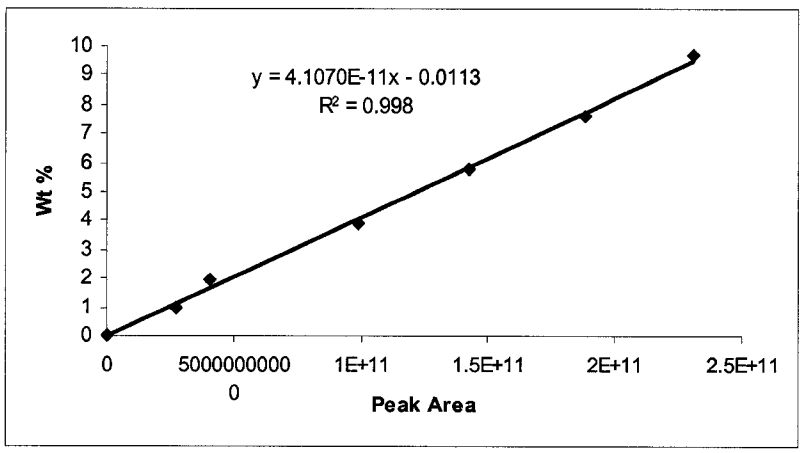
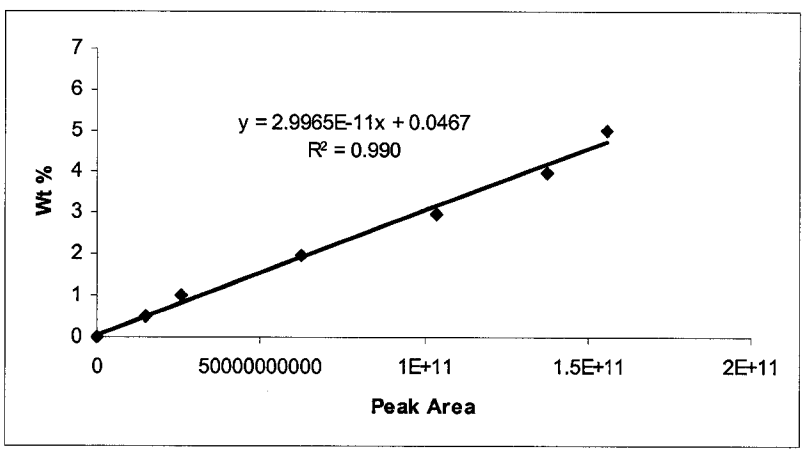
# APPENDICES

# APPENDIX A

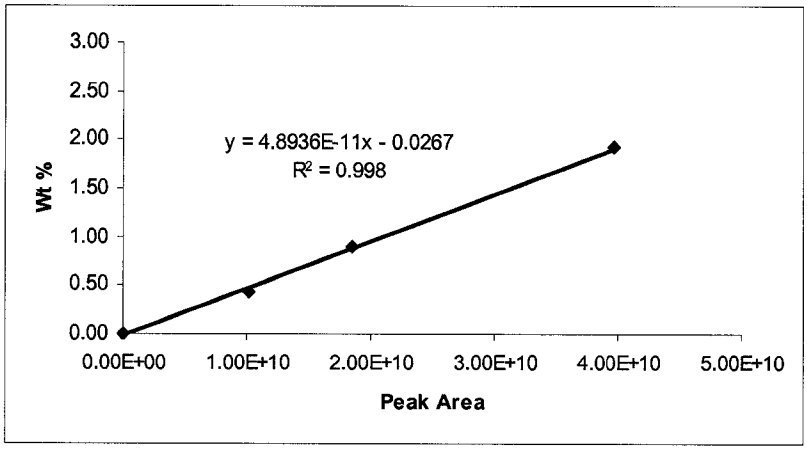
## CALIBRATION CURVES AND STANDARDS



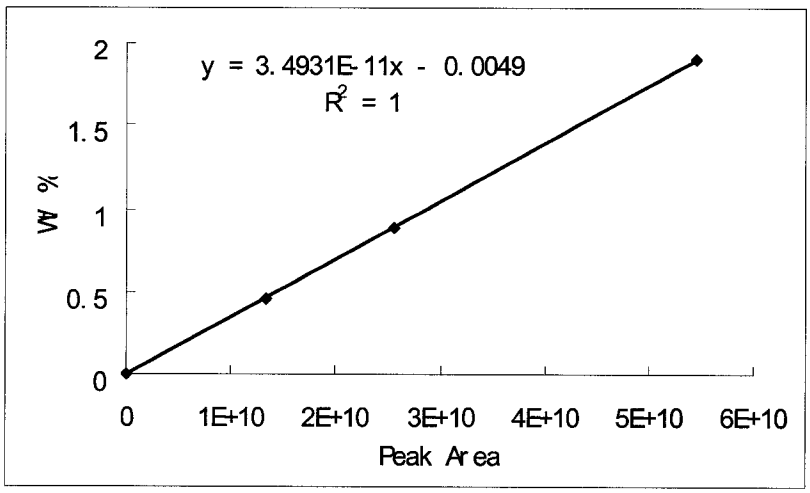

Standard curve of methyl oleate in solvent hexane.




Standard curve of methyl oleate in solvent methyl acetate.




Standard curve of methyl oleate in solvent tert-butanol.




Standard curve of ethyl oleate in solvent hexane.

## APPENDIX B

## SAMPLE CALCULATIONS

B1) **Yield of biodiesel**

a) Raw Data:

Novozym®435: 0.05g (500 U)

Methanol: 0.1228g

Used olive oil: 0.8375g

Solvent hexane: 2.6385g (4ml)

Reaction time: 48h

b) Molar ratio of methanol to triolein:

Molecular weight of methanol: 32.04 g / mol

Mol of methanol: 0.1228g / (32.04 g / mol) = 0.0038 mol

Molecular weight of triolein: 885.445 g / mol

Weight percentage of triolein in used olive oil: 45%

Mol of triolein: 0.8375g * 45% / (885.445 g / mol) = 0.00043 mol

Molar ratio of methanol to triolein: 0.0038 mol : 0.00043 mol = 9:1

c) GC analysis

Sample taken: 0.0806g

Hexane added for dilution: 0.6571g

Standard curve: y = 4.1070E-11 * x – 0.0113

y – biodiesel percentage, x – GC peak area

Diluted sample peak area: 1.80E+10

Diluted sample biodiesel weight percentage: y = 4.1070E-11 * 1.80E+10 – 0.0113 = 0.7261 %

Original sample biodiesel weight percentage: y = 0.7261 % * (0.0806g sample + 0.6571g dilution hexane) / 0.0806g sample = 6.6456 %

d) Yield of biodiesel

Total reaction mixture: 0.1228g methanol + 0.8375g used olive oil + 2.6385g hexane = 3.5988g

Biodiesel produced: 3.5988g * 6.6456 % = 0.2392 g

Yield of biodiesel: 0.2392g biodiesel / 0.8375g used oil = 0.2856 g biodiesel / g used oil

Biodiesel of 100% conversion: 0.8375g used olive oil * 45% of triolein * 889.445 / 885.445 = 0.3786g

Yield of biodiesel: 0.2392g / 0.3786g = 60.17 %

B2) **Productivity**

$$\text{Productivity} = \frac{\text{Yield} * \text{Wight of used oil (g)}}{\text{Enzyme activity (U)} * \text{Reaction time (h)}}$$

Raw data:

Yield of biodiesel: 0.290 g biodiesel / g used oil

Weight of used oil: 0.8375g

Enzyme activity: 500 U

Reaction time: 42h

Productivity = 0.290 (g biodiesel / g used oil) * 0.8375g / 500 U/ 24 h

= 1.157E-5 g biodiesel / U enzyme-h